X-Ray Crystallographic and Kinetic Studies of Human Sorbitol Dehydrogenase


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Summary

Sorbitol dehydrogenase (hSDH) and aldose reductase form the polyol pathway that interconverts glucose and fructose. Redox changes from overproduction of the coenzyme NADH by SDH may play a role in diabetes-induced dysfunction in sensitive tissues, making SDH a therapeutic target for diabetic complications. We have purified and determined the crystal structures of human SDH alone, SDH with NADH, and SDH with NAD+ and an inhibitor that is competitive with fructose. hSDH is a tetramer of identical, catalytically active subunits. In the apo and NADH complexes, the catalytic zinc is coordinated by His69, Cys44, Glu70, and a water molecule. The inhibitor coordinates the zinc through an oxygen and a nitrogen atom with the concomitant dissociation of Glu70. The inhibitor forms hydrophobic interactions to NADH and likely sterically occludes substrate binding. The structure of the inhibitor complex provides a framework for developing more potent inhibitors of hSDH.

Introduction

Diabetes mellitus afflicts approximately 151 million people worldwide, with an estimated increase to 221 million by 2010 (Zimmet et al., 2001). Diabetic individuals often suffer debilitating long-term complications such as nerve, retina, kidney, and heart damage, and have higher incidences of atherosclerosis and stroke. The molecular basis of diabetic complications is not well understood, but over the last decade, large-scale studies have shown that intensive therapy to reduce blood glucose levels can delay the onset and slow the progression of diabetic complications (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study Group, 1998). However, intensive therapy is extremely difficult to implement and is associated with significant side effects, such as increased frequency of hypoglycemia (The Diabetes Control and Complications Trial Research Group, 1993). At this time, no therapy specific for the treatment of diabetic complications is widely accepted.

A leading metabolic approach to control the detrimental effects of excess flux of glucose in diabetic tissues has been to inhibit aldose reductase (AR), the first enzyme of the polyol pathway (Figure 1A). Aldose reductase inhibitors have shown promising preclinical results in animal models of diabetic complications (Sarges and Oates, 1993) and have recently been reported to improve neuropathy and nephropathy surrogate endpoints in early clinical trials (Greene et al., 1999; Hotta et al., 2001; Iso et al., 2001). A closely related concept (Williamson et al., 1993; recently reviewed in Tilton, 2002; Oates, 2002) emphasizes the importance of the increase in the cytoplasmic free NADH/NAD+ ratio that results from excessive oxidation of sorbitol to fructose by SDH, creating a "pseudo-hypoxic" state that contributes to glucose-linked oxidative stress (Williamson et al., 1993; Tilton, 2002) and is therefore a central determinant of vascular dysfunction and eventual pathology in diabetic tissues (Rosen et al., 2001). Specific inhibitors of SDH are needed to study the role of excess flux through SDH in the development of diabetic complications.

Prototype sorbitol dehydrogenase inhibitor (SDI) CP-166,572 (SDI-158; Figure 1B) was identified in 1994 (Geissen et al., 1994) and provided the starting point from which more potent SDIs were synthesized (Mylari et al., 2001, 2002; Chu-Moyer et al., 2002). Although it has been proposed that the compound inhibits enzyme activity by directly chelating the catalytic zinc (Mylari et al., 2001; Lindstad and McKinley-McKee, 1997), a definitive proof, such as a crystal structure, has not been available. Recently, two X-ray crystal structures have contributed to our knowledge of the SDH enzymes. The 3.0 Å resolution structure of rat sorbitol dehydrogenase has been determined (Johansson et al., 2001), as has a high-resolution structure of the silverleaf whitefly NADP(H)-dependent ketose reductase (Banfield et al., 2001), which preferentially catalyzes the reduction of fructose to sorbitol. Crystals of human SDH have also been reported (Darmanin et al., 2003).

Here, we present the first X-ray crystal structures of human sorbitol dehydrogenase (hSDH, E.C. 1.1.1.14), as well as hSDH plus its required cofactor, NAD+, and a ternary complex of hSDH, NADH, and a reversible active site inhibitor, CP-166,572. These X-ray structures...
Figure 1. Substrates, Cofactors, and Inhibitors of hSDH

(A) The polyol pathway consists of two enzymes: aldose reductase (AR) and sorbitol dehydrogenase (SDH). The structures of sorbitol and fructose are shown with their respective carbon atoms numbered. The nicotinamide ring of NAD⁺ and NADH are shown, R represents the adenine dinucleotide portion of both molecules (omitted for simplicity).

(B) Prodrug SDI-157 and CP-166,572 (SDI-158 [7], WAY-135706 [32]). Structures and inhibition constants (IC₅₀) for each molecule are shown. Figures made in ChemDraw (CambridgeSoft, 2001).

provide experimental evidence that, when combined with kinetic, ligand binding, and literature data, allow us to delineate each step along the SDH reaction pathway (Figure 1A). The structure of native SDH corresponds to the “ground state” of the enzyme and reveals the ligands to the catalytic zinc in the absence of cofactor or substrate. The structure of SDH complexed with NAD⁺ reveals the surprisingly slight structural changes the protein makes when binding the cofactor in the first step of the ordered reaction. The third structure, a complex of SDH/NADH and the inhibitor CP-166,572, together with our kinetic studies showing that CP-166,572 is a competitive inhibitor with respect to fructose (but not with respect to sorbitol, NAD⁺, or NADH), allows us to propose a molecular model for (1) the binding of fructose (and sorbitol) in their linear forms, and (2) inhibition by CP-166,572, whose binding site overlaps with linear fructose but binds with greater affinity due to improved hydrophobic contacts.

Results and Discussion

Complexes Solved
We first crystallized the human enzyme as a complex with NADH and inhibitor CP-166,572 with four molecules in the asymmetric unit (Table 1). The structure was solved by the method of single anomalous diffraction (SAD) with phase information derived from the anomalous scattering of 32 selenium atoms from four molecules of selenomethionine-substituted hSDH (Table 1). Subsequently, we crystallized the enzyme alone and as a complex with NAD⁺ (Table 1).

All three complexes (native SDH, SDH/NAD⁺, and SDH/NADH/CP-166,572) crystallized in the same space
Table 1. Crystallographic Data and Refinement Statistics

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Native</th>
<th>NAD&lt;sup&gt;+&lt;/sup&gt;</th>
<th>SeSDH/NADH/CP-166,572</th>
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</thead>
<tbody>
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<td>P6&lt;sub&gt;2&lt;/sub&gt;</td>
<td>P6&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
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<td>a = b = c</td>
<td>134.95</td>
<td>133.78</td>
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<td>99.0–1.9</td>
<td>99.0–1.9</td>
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<td>161,025</td>
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<tr>
<td>Redundancy</td>
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<td>4</td>
<td>&gt;9</td>
</tr>
<tr>
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<td>1.0 (0.91)</td>
<td>1.43 (0.96)</td>
</tr>
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<td>0.083 (0.379)</td>
<td>0.124 (0.581)</td>
</tr>
<tr>
<td>I/error</td>
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<td>12.8 (2.0)</td>
<td>33.6 (1.8)</td>
</tr>
<tr>
<td>Completeness</td>
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<td>0.886 (0.442)</td>
<td>0.996 (0.883)</td>
</tr>
</tbody>
</table>

Phasing Statistics

- Phasing power (friedels): 1.24
- FOM (overall): 0.27
- R<sub>rank</sub>: 0.73
- Mean FOM (after solvent flipping [CNS]): 0.99
- Solvent content: 0.65

Refinement Statistics

- Resolution (Å): 43.2–2.2, 45.8–1.9, 57.8–2.0
- R: 0.200 (0.254), 0.195 (0.270), 0.183 (0.244)
- R<sub>free</sub>: 0.233 (0.279), 0.222 (0.291), 0.211 (0.221)
- R<sub>rms</sub> bond lengths (Å): 0.006, 0.005, 0.008
- R<sub>rms</sub> bond angles (°): 1.3, 1.4, 1.5
- Protein: 1–356, 1–356, 1–356
- Ligands: 4 Zn, 4 Zn, 4 Zn/NAD<sup>+</sup>, 4 Zn/NADH/4 CP-166,572

Resolution, chi<sup>2</sup>, Rmerge, I/error (Intensity divided by error), and completeness values taken from Scalepack results (Otwinowski and Minor, 1997). R<sub>merge</sub> = \[ \frac{\sum |I_{h}(i) - \langle I_{h} \rangle|}{\sum I_{h}(i)} \] where \( I_{h}(i) \) and \( \langle I_{h} \rangle \) are the i<sup>th</sup> and the mean measurement of the intensity of reflection \( h \). R<sub>cryst</sub> = \[ \frac{\sum |F_{o}(h) - F_{c}(h)|}{\sum F_{o}(h)} \] where \( F_{o}(h) \) and \( F_{c}(h) \) are the observed and calculated structure factor amplitudes. R<sub>free</sub> is computed using 10% of the reflections from each resolution shell, randomly selected.

Overall Description of the Enzyme

SDH is a tetramer (Jeffrey et al., 1981; Banfield et al., 2001; Johansson et al., 2001), with each subunit consisting of 356 residues (38 kDa) and one catalytic zinc atom (Jeffrey et al., 1984; Maret, 1996). The four subunits are related by crystallographic and noncrystallographic symmetry and can be considered identical within the resolution of the data (see Experimental Procedures). The hSDH tetramer can be considered (like other medium-chain dehydrogenase/reductase family members; Ruzeinikov et al., 2001) as a dimer of identical dimers (dimer AD and dimer BC, Figure 2A). The largest dimer interface is formed between the A and D (or equivalent B and C) subunits, burying a total of 2850 Å<sup>2</sup> of solvent-accessible surface in the native structure, more than twice the interface area formed by subunits A and B within the tetramer (1233 Å<sup>2</sup>). In forming the tetramer, the AD dimer contacts the BC dimer at two regions, sequestering a total of 7510 Å<sup>2</sup> of solvent-accessible surface per dimer.

The N-terminal seven residues of the protein provide a striking crystal contact (Figure 2B). These residues pack between two β strands of a crystallographic symmetry mate (strand residues 321–328 and 352–356 of subunit B), forming a link between the two B subunits from different tetramers. In solution, residues 1–7 are expected to pack against β strands from their own subunit (residues 24–31 and 72–79). This alternate conformation of the N terminus may be required to stabilize the lattice, which has a solvent content of 65%.

The overall fold of one subunit of hSDH is similar to that of rat SDH (coordinates not available), as expected, but is also closely related to the folds of NADPH-dependent whitefly ketose reductase (rmsd of 1.3 Å for 345 C<sub>α</sub> carbons) and horse liver alcohol dehydrogenase (ADH, rmsd of 1.8 Å for 301 C<sub>α</sub> carbons; 8ADH.pdb). The hSDH monomer consists of two β barrel domains with a deep cleft between them (Figure 2B). The active site is located at the bottom of the cleft facing the surface of the tetramer. The coenzyme binding domain (residues 158–298) is a classic Rossmann fold (Rossmann et al., 1974) that reversibly binds the required NAD(H). The coenzyme binds identically and with full occupancy in all subunits.
Figure 2. Overall View of hSDH

(A) Biological tetramer of human sorbitol dehydrogenase. A ribbon diagram of the tetramer coordinates derived from complex of hSDH with zinc, NADH, and CP-166,572, which are shown as stick figures. Subunit A is colored green, B is blue, C is yellow, and D is red. All figures prepared using Ribbons (Carson, 1991) with atoms colored as follows: zinc, purple; carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow; and water molecules, red spheres.

(B) Structure of the monomer of human sorbitol dehydrogenase. Ribbon diagram showing the overall fold of one subunit of human sorbitol dehydrogenase and the location of CP-166,572, NADH, and the catalytic zinc atom.

of the tetramer. The larger catalytic domain (residues 1–157, 299–356) houses the single zinc atom required for activity. hSDH does not contain a second structural zinc atom like alcohol dehydrogenase (Eklund et al., 1976) or the whitefly NADP(H) ketose reductase (Banfield et al., 2001).

Stoichiometry and Michaelis Constants of hSDH Ligands

Human SDH catalyzes the reversible oxidation of the C2 carbon of sorbitol to fructose with reduction of \( \text{NAD}^+ \) to NADH (the “forward” direction, left to right in Figure 1A). SDH also readily catalyzes the reduction of fructose...
to sorbitol (“reverse” direction, Figure 1A). The equilibrium constant for sheep liver SDH is 3.7 nM (Lindstad et al., 1992) and, because a proton is part of the mass balance equation together with fructose and NADH, low pH facilitates fructose reduction to sorbitol, while high pH favors sorbitol oxidation to fructose (Lindstad and McKinley-McKee, 1995). Studies with the enzyme from sheep liver have demonstrated that the kinetic mechanism is compulsory ordered bi-bi with the coenzyme binding first and leaving last (Lindstad et al., 1992). To confirm that all four coenzyme binding and active sites with sorbitol binding dependent on the presence of NAD(H) or other ligands.

We also determined the Michaelis constants of several key substrates and coenzymes for hSDH. The human enzyme exhibits equal apparent affinities ($K_m$) for NAD$^+$ and NADH: $225 \pm 12 \mu$M (n = 3) and $210 \pm 61 \mu$M (n = 3), respectively. In the presence of 1 mM NAD$^+$, the $K_m$ for sorbitol was $1.5 \pm 0.3 \mu$M (n = 5), a value indistinguishable from that for rat SDH (Oates, 2002). In the “forward” direction, the product fructose exits first, followed by the rate limiting release of NADH (Lindstad et al., 1992). In the presence of 0.5 mM NADH (a saturating concentration of NADH was used because NADH absorbs strongly at 340 nm, the wavelength used to monitor the reaction), the $K_m$ for fructose is $270 \pm 63$ nM (n = 3). It should be noted that the measured $K_m$ for fructose can be misleading, since only 0.8% of the substrate form (Maret, 1996). Adjusting the measured $K_m$ by this factor gives a corrected $K_m$ of $2.2 \mu$M, close to the 1.5 mM $K_m$ measured for sorbitol. Consistent with the observations of Maret and Auld (1988), we did not find evidence of substrate inhibition with the human enzyme.

**Zn Ligands in hSDH**

In the crystal structure of hSDH alone, each catalytic site is marked by the presence of a zinc atom. Each zinc atom is coordinated by interactions with Cys44, His69, Glu70, and a water molecule (W1952, Figure 3B, Table 2), but the angles vary from 96° to 120° and are therefore not strictly tetrahedral. In contrast to suggestions from mutagenesis data (Karlsso and Hoog, 1993) and by the 3.0 Å structure of rat SDH (Johansson et al., 2001), the nearby Glu155 is not a direct ligand to the zinc atom. Instead, Glu155 is linked to the zinc atom by the 3.0 Å structure of rat SDH (Johansson et al., 2001).
through the aforementioned water molecule and thus belongs to the second coordination sphere. The structure of whitefly NADP(H) ketose reductase shows a very similar coordination of the catalytic zinc with Cys41, His66, Glu67, and a water molecule linked through Glu152 (Banfield et al., 2001). It is possible that, like Glu68 in ADH, Glu155 may transiently coordinate directly to the zinc atom to permit release of the product during catalysis, as proposed from a computational analysis (Ryde, 1995).

Changes in hSDH on Binding NAD$^+$

The coenzyme fits neatly into a surface crevice formed by loops emanating from the six $\beta$ strands forming the core of the coenzyme binding domain. The conformation of NAD$^+$ may be compared to the conformation of NADH bound to mammalian ADH (2OHX.pdb) in the absence of a binary complex of ADH with NAD$^+$. Both coenzymes adopt nearly identical extended conformations with the ribose in the 2'$\pi$ endo puckering conformation and the nicotinamide ring anti to the ribose (Figure 3C).

In the ordered reaction mechanism, NAD$^+$ binds first (Figure 3A), and there are small but significant changes in the conformation of a handful of residues to accommodate its bulk (Figure 3C). In contrast, liver alcohol dehydrogenase undergoes a significant domain rearrangement on binding its cofactor (Eklund and Branden, 1979). The domain orientation of hSDH is closest (rmsd of 1.3 Å for 345 Cα carbons) to that of the whitefly NADP(H)-dependent ketose reductase (Banfield et al., 2001), which lacks coenzyme and differs in complicated ways from both apo ADH (rmsd of 1.8 Å for 301 Cα carbons; 8ADH.pdb) and the ADH/NADH complex (rmsd of 1.9 Å for 270 Cα carbons; 2OHX.pdb). Both the NAD$^+$ and NADH/SDI complexes were determined from co-crystals rather than by soaking ligands into an existing crystal lattice, suggesting that the domain orientations in the crystal are biologically relevant. However, it is possible that an alternative domain rearrangement, like that seen in ADH on binding NADH, occurs in solution but is not compatible with the crystal lattice shared by all three structures or does not occur at the pH of crystallization (6.2).

Comparing the coordinates of SDH with and without NAD$^+$, the side chains of Arg208, Leu274, and Val296 move to relieve steric clashes with different parts of the NAD$^+$ molecule. Of these, the most dramatic change is the movement of the side chain of Arg208, which breaks an electrostatic interaction with Asp203 and flips 180° about Cα to neutralize one of the phosphate oxygens on NAD$^+$. In addition, the backbone atoms of Ile183 and the carbonyl of Cys249 move to expand the NAD$^+$ binding pocket. Comparison of the two structures shows there are no changes in the residues coordinating the zinc atom.

The sequence and structural basis for the selection of NAD$^+$ over NADP$^+$ in dehydrogenases has been described (Baker et al., 1992), and hSDH fits the known pattern. The primary determinant of NAD$^+$ dependency is the presence of an aspartic acid (in hSDH, Asp203), which forms hydrogen bonds to the 2' and 3' hydroxyls of the NAD$^+$ ribose and occupies the space that would be occupied by the phosphate attached to the 2' hydroxyl in NADP$^+$. Dehydrogenases that use NADP$^+$ have an alanine at this position, which is small enough to leave room for the phosphate. Whitely ketose reductase uses NADP$^+$ as its cofactor and has the requisite alanine (residue 199) instead of aspartic acid. Although the structure lacks coenzyme, a phosphate molecule from the crystallization buffer was found adjacent to alanine 199 (Banfield et al., 2001). Superposition of the whitefly ketose reductase coordinates with those of the SDH/ NAD$^+$ structure shows that this phosphate lies within covalent bonding distance of the 2' OH of the ribose of NAD$^+$, exactly where it should be, if NADP$^+$ were bound.

hSDH Inhibitor CP-166,572

There is only one class of demonstrated in vivo active sorbitol dehydrogenase inhibitors, the piperazine pyrimidines, discovered at Hoechst (Geissen et al., 1992). A second class, consisting of dithiothreitol and other metal chelating thiols, are also potent in vitro inhibitors by virtue of their interactions with the catalytic zinc [Lindstad and McKinley-McKee, 1996]. SDI-157 was initially described as a sorbitol-raising compound of unknown mechanism, but was soon found by Geissen (Geissen et al., 1994) and others (Oates et al., 1994) to be a prodrug that gave rise to an active SDI, CP-166,572, also called SDI-158 or WAY 135,706 (Cameron et al., 1997) (Figure 1B). SDI-157 and CP-166,572 provide a dramatic example of the pharmacological power of a small chemical change: in vivo hydroxylation of the methyl group of the pyrimidine ring of SDI-157 results in CP-166,572 and a 1800-fold drop in the IC$_{50}$ to 0.25 μM (Geissen et al., 1994). The $K_c$ of CP-166,572 for human SDH is 154 ± 22 nM (mean ± standard deviation, n = 3, unpublished data). CP-166,572 is the prototype of this class and is selective for SDH against other dehydrogenase en-

Table 2. Zinc Ligands in Each Structure

<table>
<thead>
<tr>
<th>Native</th>
<th>NAD$^+$</th>
<th>SeSDH/NADH/CP-166,572</th>
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<tr>
<td>Cys44:S$^\gamma$</td>
<td>2.4</td>
<td>Cys44:S$^\gamma$</td>
</tr>
<tr>
<td>His69:N$^\epsilon$</td>
<td>2.2</td>
<td>His69:N$^\epsilon$</td>
</tr>
<tr>
<td>Glu70:O$\gamma$</td>
<td>2.3</td>
<td>Glu70:O$\gamma$</td>
</tr>
<tr>
<td>WAT 1952*</td>
<td>2.3</td>
<td>WAT 1952*</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>CP: N1</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>CP: O30</td>
</tr>
</tbody>
</table>

Distances between the atom and the catalytic zinc atom are given in angstroms. Residues within 2.5 Å are presumed to be coordinated directly to the zinc atom. Residues too far to coordinate directly to the zinc atom are in italics. Waters marked with an asterisk are structurally equivalent.
zymes (Geissen et al., 1994), including lactate, alcohol, fructose, and glyceraldehyde-3-phosphate dehydrogenase, even at concentrations up to 50 μM (Oates et al., 1994). We observed that inhibition of hSDH by CP-166,572 was uncompetitive with respect to NAD⁺, sorbitol, or NADH (Figures 4A–4C). Inhibition of sheep SDH by CP-166,572 with respect to fructose has been reported to be competitive (Geissen et al., 1994) or mixed noncompetitive (Lindstad and McKinley-McKee, 1997). We observed both patterns (Figures 4D and 4E) and noted that the discrepancy appears to be a function of the NADH concentration used, with 500 μM NADH (a concentration above the Kₘ) giving the competitive pattern (Figure 4D) and 50 μM NADH (less than the Kₘ) exhibiting a mixed noncompetitive pattern (Figure 4E). At 500 μM NADH, CP-166,572 also inhibited sheep SDH competitively with respect to fructose (Figure 4F). Thus, under these conditions, CP-166,572 binds to the SDH-NADH complex in a manner competitive with fructose in both human and sheep SDH.

The crystal structure of hSDH/NADH/CP-166,572 shows that CP-166,572 is positioned such that the pyrimidine ring makes contacts with both the zinc atom and NADH, while the piperazine ring and its substituents extend out toward the surface of the protein (Figures 5A and 5B). CP-166,572 inhibits substrate binding by chelating the catalytic zinc ion through strong interactions with both the N1 nitrogen and the O30 oxygen of the hydroxymethyl. The pyrimidine ring of CP-166,572 stacks over the nicotinamide ring of NADH, forming a tight hydrophobic contact. The inhibitor CP-166,572 binds more tightly than either sorbitol or fructose (submicromolar and low millimolar Kₘ’s, respectively) because the inhibitor pyrimidine ring stacks optimally with NADH while simultaneously presenting O30 and N1 at a fixed distance for coordination to the catalytic zinc.

Although NAD⁺ does not alter the constellation of amino acid residues coordinating the catalytic zinc, the inhibitor CP-166,572 does. Glu70 is replaced by two ligands from the inhibitor, making a total of five coordination sites (with angles ranging from 74° to 112°), rather than the four found in the absence of CP-166,572. Both the nitrogen N1 of the pyrimidine ring and oxygen O30 of the hydroxymethyl group of CP-166,572 displace water molecules to chelate the zinc atom as previously deduced from structure-activity relationships (SAR) of isomeric pyrimidines (Mylari et al., 2001). Displaced from the zinc atom, Glu70 forms a hydrogen bond to a water molecule (W1064, the same one that forms a hydrogen bond to Glu155 in the NAD⁺ complex). After superimposing the coordinates of the SDH/NAD⁺ structure onto the SDH/NADH/CP-166,572, the zinc atom is displaced 2.3 Å toward the inhibitor in the CP-166,572 complex. There are also significant changes in the main chain and side chain atoms of residues 42–46, most notably rotation of the side chain of Cys44 to maintain the optimal distance between S₁ and the zinc atom in each structure.

The strength of the link between the zinc atom and the oxygen of the hydroxymethyl moiety of CP-166,572 depends on the ability of the oxygen to donate its lone pair of electrons. The availability of these electrons depends on the extent to which their attendant hydrogen is engaged in other interactions. Potential hydrogen bonds could be formed between the oxygen atom and His69:Nε2 (3.0 Å) or Glu155:Oε2 (2.4 Å). Based on the distance and because His69 is a zinc ligand, Glu155 is likely the hydrogen bond acceptor. With the hydrogen shared by another atom, the lone pair of electrons on the oxygen is more available for interaction with the zinc atom.

In addition to coordinating the zinc, the inhibitor is stabilized in the catalytic site by numerous hydrophobic interactions (Figure 5C). Important stacking interactions with ideal distances between 3.6 and 4.3 Å are achieved between the pyrimidine ring of CP-166,572 and the nicotinamide ring of NADH. The presence of this hydrophobic interaction suggests a reduced affinity of the enzyme for the inhibitor in the absence of the NADH coenzyme, as well as a relatively reduced affinity in the presence of the charged NAD⁺ form of the bound coenzyme. The inhibitor is also surrounded by hydrophobic residues on both sides, which pack closely against it (Tyr50, Ile56, Phe59, Phe118, Thr121, Leu274, Phe297, and the aliphatic part of Arg298). This finding accounts for the remarkable potency of analogs in which the sulfamoyl group is replaced by planar rings substituted with hydrophobic groups, such as methyl (Mylari et al., 2002).

Aside from the interaction made with the oxygen O30 described above, no other direct hydrogen bonds are formed between the inhibitor and the protein. One potential water-mediated hydrogen bond is formed between the pyrimidine nitrogen N3 and the guanidinium group of Arg298. Water molecules are recruited from those occupying the catalytic site to satisfy the hydrogen bonding potential of inhibitor atoms, which are too far to interact with protein residues. For example, both oxygens of the sulfate group form hydrogen bonds to water molecules and are accessible to bulk solvent.

This structure refines previous structural proposals based on models of ADH (Lindstad and McKinley-McKee, 1997; Darmanin and El-Kabbani, 2000). In the first, a hydrogen bond between the hydroxyl group of CP-166,572 and either Glu154 (Glu155 in hSDH) or Lys293 (Lys298 in hSDH) was suggested, with the N3 nitrogen of CP-166,572 coordinated to the catalytic zinc (Lindstad and McKinley-McKee, 1997). However, as indicated earlier, SAR obtained from other pyrimidine stereoisomers of CP-166,572 suggested that the N1 rather than N3 served as a ligand to the zinc (Mylari et al., 2001). The second proposal, based on a model of human SDH derived from structures of alcohol dehydrogenases, is incorrect, in part because the side chain positions differ significantly from the crystal structure of hSDH and the observed stacking interaction between CP-166,572 and the nicotinamide was not predicted (Darmanin and El-Kabbani, 2000).

Model of Fructose Bound to hSDH

Our kinetic analysis revealed that fructose but not sorbitol competes with CP-166,572 (Figures 4D and 4F; Geissen et al., 1994), suggesting that the inhibitor and substrate binding sites overlap, at least in part (Figure 6). Like aldose reductase, which acts on the open chain form of glucose (Imagaki et al., 1982), SDH likely utilizes
Figure 4. Inhibition of hSDH by CP-166,572

Experimental details as described in Experimental Procedures.

(A) Uncompetitive inhibition of hSDH by CP-166,572 with respect to NAD$^+$: Forward direction, human SDH.

(B) Uncompetitive inhibition of hSDH by CP-166,572 with respect to sorbitol. Forward direction, human SDH.

(C) Uncompetitive inhibition of hSDH by CP-166,572 with respect to NADH. Reverse direction, human SDH.

(D) Competitive inhibition of hSDH by CP-166,572 with respect to fructose. Reverse direction, human SDH (500 μM NADH).

(E) Mixed noncompetitive inhibition of hSDH by CP-166,572 with respect to fructose. Reverse direction, human SDH (50 μM NADH).

(F) Competitive inhibition of hSDH by CP-166,572 with respect to fructose for sheep SDH. Reverse direction, sheep SDH (500 μM NADH).
the straight chain keto form of fructose for reduction to sorbitol at the time of hydride delivery from NADH (Maret, 1996). The presumed driving force for this reduction comes from zinc polarizing the C2 hydroxyl, as has been suggested for other zinc-mediated reductions. In the absence of crystallographic data for substrate binding to any sorbitol dehydrogenase, we developed a model of fructose binding using the binding mode we observed for CP-166,572, i.e., with the zinc penta-coordinated. Zinc is usually coordinated to 4 or 5 ligands in the active sites of enzymes, including bound water molecules, inhibitors, or intermediates (Lipscomb and Strater, 1996).

We manually positioned a linear fructose molecule such that both the C1 hydroxyl and C2 carbonyl oxygens were in direct coordination with the zinc atom, overlapping with the O30 hydroxyl and the N1 nitrogen, respectively, of the inhibitor. The carbons of fructose were roughly aligned with the pyrimidine and piperazine rings of the inhibitor to optimize hydrophobic interactions. The coordinates of CP-166,572 were replaced with those of fructose, and the complex of SDH/NADH/fructose was subjected to minimization in the absence of X-ray data using CNS (Brunger et al., 1998).

After minimization, the O1 and O2 oxygens of fructose are directly coordinated to the catalytic zinc atom. O1 also potentially makes two hydrogen bonds to Glu155:Oe2 and/or His69:N=2. In this position, the C2
carbon of fructose is within van der Waals distance of the reactive carbon of NADH and carbons C3, C4, and C5 are packed against the nicotinamide ring. We presume that the remaining hydroxyls of fructose will be solvated by the numerous water molecules in the catalytic site. In addition, Arg298 is likely to alter its side chain conformation to form a hydrogen bond to the O3 or O6 oxygen. An important component of the binding energy comes from hydrophobic interactions between fructose and NADH. The C2 to C5 carbons of the fructose stack against the planar hydrophobic nicotinamide ring, reminiscent of the way the aliphatic portions of arginine side chains stack under adenine rings in DNA/protein complexes.

Proposed Mechanism
To summarize, we propose the following structural changes as catalysis occurs in the forward direction (Figure 6B). In the absence of any ligand, the zinc atom is tetra-coordinated by three protein side chains (Cys44, His69, Glu70) and one water molecule (Figure 6B (1)). The required cofactor, NADH, binds first, and small but significant movements in main chain and side chain atoms occur to accommodate it, followed by the binding
of sorbitol. Sorbitol binds with both its C1 and C2 oxygen atoms coordinated to zinc with the simultaneous release of Glu70, making the zinc pentavalent (Figure 6B (2)).

The backbone carbons C3–C5 of sorbitol overlap with the linear fructose model herein proposed (Figure 6A). The carbon backbone of sorbitol stacks in part against the nicotinamide ring, an interaction that is consistent with the observed ordered mechanism.

Oxidation of sorbitol to fructose in the enzyme-substrate complex now formed requires a base to remove the hydrogen from the C2 oxygen of sorbitol coordinated to zinc. One candidate for this role is water molecule W1952, which forms a hydrogen bond to Glu155 in the apo enzyme and to both Glu155 and Glu70 in the complexes with the inhibitor and our model for fructose. This water molecule is proposed to function as a general base, which abstracts the proton of the zinc-coordinated C2 hydroxyl, setting up the cascade of events to deliver the hydrate to C2 to NAD$^+$ with concomitant charge neutralization of NAD$^+$ to NADH and oxidation of the C2 hydroxyl to the keto group of fructose (arrows, Figure 6B (2)). The reverse flow of electrons from NADH to reduce fructose to sorbitol is depicted in Figure 6B (3, arrows). In the forward direction (Figure 6B (4, via 3A)), since fructose blocks the exit of NADH from the protein, fructose must be released first, followed by the release of NADH and the subsequent return of residues around the catalytic zinc to their original tetra-coordinated configuration.

A key issue is whether the fructose and sorbitol substrates bind to the enzyme in precisely the same way CP-166,572 does. In the present case, speculations on this point are subject to the caveat that the enzyme crystals were grown at pH 6.2 and the results may differ from what occurs at neutral pH, the conditions under which the enzyme kinetic studies were performed. In the absence of definitive data that will come only from the solution of additional crystal structures, we can nevertheless note that in the presence of 500 μM NADH, fructose competes with CP-166,572 (Figures 4D and 4F); this implies, but does not prove, that the latter two ligands have one or more binding sites on the enzyme surface that overlap. On the other hand, in the presence of the inhibitor, the catalytic zinc is coordinated by only two protein ligands plus a water molecule (Figure 5B). This coordination state is unusual; in all other zinc enzymes, the catalytic zinc is coordinated by three amino acid side chains with either a water molecule or another side chain as the fourth ligand. It is therefore possible that the fructose and sorbitol substrates do not bind like CP-166,572, but instead interact with the zinc through a single coordinating oxygen, e.g., the C2 hydroxyl. It should be noted that coordination by either a single substrate oxygen or by two substrate oxygens does not preclude the occurrence of a competitive inhibition pattern between CP-166,572 and fructose but not between CP-166,572 and sorbitol (Figures 4B and 4D; Geissen et al., 1994).

One factor which could explain the inhibition pattern of CP-166,572 is that the neutral nicotinamide ring of the NADH cofactor may form an important part of the hydrophobic interactions in the binding site for fructose as it does for CP-166,572 (in the case of CP-166,572 via the neutrally charged pyrimidine ring of the inhibitor [Figures 5C and 6A]); hence, CP-166,572 and fructose would compete with one another. In contrast, the positively charged nicotinamide ring of NAD$^+$ (which exists only in the presence of sorbitol) may better accommodate the pyrimidine ring of CP-166,572 in the charged state. Thus, CP-166,572 might compete effectively with substrate only when NADH, but not NAD$^+$, is bound.

An alternative possibility is that CP-166,572 competes (only) with the furanose form of fructose on the enzyme surface and not with the linear form of this ketose (Figure 6B (4)). Further studies will be necessary to resolve these and related questions, such as the actual coordination state of zinc in the presence of sorbitol and fructose substrates.

In summary, we have purified, crystallized, and determined the crystal structures of human SDH in its native state, as a complex with NAD$^+$ and as a ternary complex with NADH and a prototype inhibitor, CP-166,572. These structures provide a view of the reaction pathway during catalysis and a firm foundation for understanding the mechanism of action of reversible inhibitors of SDH.

Experimental Procedures

Cloning and Expression of Human SDH

A pET vector system (Novagen) was used for expression of human SDH. The SDH gene previously identified in a human liver cDNA library (Iwata et al., 1995) was kindly provided by Dr. Jan-Olov Höög (Karolinska Institute, Stockholm, Sweden) and was used as a template for polymerase chain reaction (PCR) amplification. Two primers were designed to amplify the DNA fragment coding for hSDH. The S′ primer (5′-GGAATTCATATGGCGGCGGCGGCCAAGCCC-3′) introduced a unique NdeI site, while the 3′ primer (5′-GGCCGCTCGAGCTTATTAGGATTCGACTCTGGA-3′) introduced a unique XhoI site preceded by TAA and TAG stop codons. PCR amplification was carried out under standard conditions using Vent polymerase and the human liver cDNA library as a template. The PCR-amplified product was digested with NdeI and XhoI, gel purified, and ligated into pET23a (Novagen) and transformed into E. coli DH5α cells. Positive clones were selected and plasmids subjected to restriction digestion and PCR analysis to confirm the presence of the insert. The DNA sequence of the 1071 base pair insert in pET23a was confirmed (identical to the human SDH sequence GenBank accession number U07361) on both strands.

Cell Growth and Initial Expression

E. coli BL21(DE3)/pET23a,1/3-hSDH-MCB3 was cultured in a media comprising 10 g/l dextrose, 6 g/l NaHPO4, 3 g/l KH2PO4, 1 g/l NH4Cl, 0.5 g/l NaCl, 5 g/l Casamino Acids, 0.5 g/l MgSO4·7H2O, 10 mg/l CaCl2, 1 mg/l thiamine, 0.5 mM l-P-2000, and 25 mg/l ampicillin-3H2O at 30°C until OD at 550 nm reached between 1.0 and 1.5. Then, 1 mM of IPTG was added and temperature reduced to 25°C. The pH during the IPTG induction phase was controlled above 6.6 using NaOH. The induction phase was allowed to go on for about 24 hr to maximize the expression of soluble, active hSDH. The culture broth was then chilled to 10°C, and cells containing soluble, active hSDH were pelleted by centrifugation.

Growth of Selenomethionine-Substituted hSDH

A glycerol stock of E. coli BL21 (DE3) cells harboring hSDH/pET23a 1/3 was cultured overnight at 37°C with shaking at 300 rpm in 2 × YT media (16 g Bacto-Tryptone, 10 g yeast extract, 5 g NaCl, in 1 l water) containing 100 μg/ml carbenicillin. Plasmid DNA was prepared from these cells (Qiagen), transformed into BL21(DE3) cells (Novagen), and used for expression in selenomethionine media.

Selenomethionine (SeMet) media contains 10 g/l (NH4)2SO4, 2.16 g/l Na2HPO4, 1.28 g/l KH2PO4, 5 g/l NaCl, 0.4 g/l trisodium citrate (Sigma C-7254), 0.2 g/l MgSO4, 2 mg/l thiamine, 10 g/l glycerol, 0.1
g/l carbencillin, 40 mg/l of all L-amino acids except methionine, 100 mg/l D, L-selenomethionine (Bachem, Cat#: GSEL20), 10 mg/l CaCl2, 4 mg/l H3BO3, 1.71 mg/l MnSO4, 2 mg/l ZnSO4, 0.373 mg/l CuSO4, 0.4 mg/l CoCl2, 0.2 mg/l Na2MoO4, adjusted to pH 7.0. A 500 ml culture of the hSDH clone in B834(DE3) in SeMet media was inoculated from an overnight culture grown at 30°C in the same media and grown to an OD600 of 0.5 to 1.0, at which point the cells were induced with IPTG (1.0 mM final concentration). After growth for 24 hr at room temperature, the cells were harvested by centrifugation and stored at –80°C.

Purification of Recombinant Human Sorbitol Dehydrogenase from E. coli Cells

The method was modified from a published procedure (Maret and Auld, 1988). E. coli (40–50 g) BL21DE3 pET 23a/hSDH cells were resuspended with two volumes of lysis buffer (20 mM HEPES/NaOH [pH 8], 5 μM ZnCl2, 2 mM DTT, 1 mM PMSF, 20 μg/ml aprotinin). Two volumes of lysis buffer containing 2 mg/ml lysozyme were added, and the mixture was stirred on ice for 60 min followed by sonication. The sonicated cells were centrifuged at 12,000 rpm for 10 min at 4°C, the supernatant was decanted, and the pellet discarded.

Ammonium sulfate was added to the supernatant to a final concentration of 40%, and the solution was placed on ice for 30 min and then centrifuged at 12,000 rpm for 10 min. The supernatant was removed and ammonium sulfate was added to a final saturation of 60%, incubated on ice for 30 min, and then centrifuged as before. The 40%–60% ammonium sulfate pellet was dissolved with 100 mM 20 mM HEPES/NaOH [pH 8.0], 5 μM ZnCl2, 2 mM DTT, 1 mM PMSF, and 1 μg/ml each of aprotinin, pepstatin, and leupeptin and dialyzed overnight at 4°C against the same buffer.

Green A Chromatography

A Green A dye column (XX-50, Pharmacia) was equilibrated with fructose (to NADH/SDH mixtures) solutions. The rate of reaction and stored at –80°C.

Mono S Chromatography

The dialyzed material was loaded onto a Mono S column (Pharmacia) equilibrated in Buffer A at 4°C against the same buffer. The column was eluted with a 0–1 M NaCl gradient in Buffer A. Four minute fractions were collected and assayed for activity. The major peak of activity, which eluted between 150–300 mM NaCl, was pooled, concentrated to 50 ml in an Amicon cell using a YM-50 membrane, and dialyzed overnight at 4°C against Buffer A.

Enzyme Kinetic Inhibition Patterns with CP-166,572

Two hundred microliters of 25 mM HEPES/NaOH [pH 7.0] were added to each well of a 96-well microtiter plate, and 25 μl of NAD+ or 5 mM NADH was added as appropriate followed by a 10 min incubation at room temperature. The reaction was initiated by the addition of 25 μl of varying concentrations of sorbitol (to NAD+/SDH mixtures) or fructose (to NADH/SDH mixtures) solutions. The rate of reaction was measured by monitoring the absorbance at 340 nm for 3 min at room temperature. Data were analyzed via nonlinear (hyperbolic) regression using GraphPad Prism Software (GraphPad Prism, Inc., San Diego, CA).

Crystalization of hSDH Protein

Both the wild-type and selenomethionine-substituted proteins were concentrated to 2.0 mg/ml in a 20 mM HEPES/NaOH (pH 7.8) containing 100 mM NaCl, 2 mM DTT, 0.1 mM NADH, and 0.2 mM of CP-166,572. Crystals were grown in hanging drops by vapor diffusion at 22°C. The well solution consisted of 150 mM NH4OAc, 100 mM Na2Citrate (pH 6.15), 30% PEG-4000, and 2.5 mM DTT. Six microliters of protein solution were mixed with 3 μl of well solution. Under these conditions, large (0.3 x 1.0 mm) crystals appeared in 4 to 8 days.
Crystals of SDH without NADH or inhibitor were prepared in the same way, except the well solution contained 28% PEG-4000 and 10 mM ZnCl₂. Crystals of SDH with NAD⁺ were prepared in the same way except the protein solution contained 0.2 mM NAD⁺ and the well solution contained 26.5% PEG-4000 and 10 mM ZnCl₂.

Stabilization of the Crystals prior to Freezing

Prior to data measurement, crystals were stabilized by stepwise transfer into a cryostabilization solution (30% PEG-4000, 37.5 mM NH₄OAc, 50 mM NaCitrate [pH 6.15], 8.75% glycerol, 0.07 mM NADH, and 0.14 mM CP-166,572), after which they were frozen either directly in a liquid nitrogen gas stream or in liquid propane.

Data Measurement and Reduction

For phasing, the data were measured from a single crystal at 100 K at beamline 5.0.2 at the Advanced Light Source at the Lawrence Berkeley Laboratory (Berkeley, CA). The X-ray absorption spectrum around the K edge of the anomalously scattering selenomethionine atoms was measured using a simple X-ray detector mounted at right angles to the X-ray beam and in the horizontal plane to capture fluorescence as an indirect measure of absorption. The peak wavelength was identified as 0.97949 Å from the fluorescence scan. A complete data set to a maximum resolution of 1.9 Å was measured using a ADSC Quantum 4 detector. For each wavelength, a 90° wedge of data was measured, after which phi was rotated by 180° and the same wedge of data remeasured to collect the Bijvoet pairs. The data were reduced and scaled using Denzo and Scalepack (Otwinowski and Minor, 1997), keeping the Bijvoet pairs separate. Data sets of the complexes with NAD⁺ and NADH/CP-166,572 were measured from a single crystals at 100 K at beamline X12C at the National Synchrotron Light Source at the Brookhaven National Laboratory (Upton, NY), using the Brandeis B4 or B1.2 detectors (Phillips et al., 2000).

Structure Solution

Data were processed in space groups P6₃ and P6₂22/P6₃22. Analysis of the native Patterson map indicated translational noncrystallographic symmetry (NCS) parallel to the c axis, with a fractional translation vector of (0,0,0.4167), suggesting that the lower symmetry in combination with translational NCS was likely. However, equivalent data reduction statistics were obtained with both symmetries (P6₃; for all data from 99–1.9 Å, Rmerge = 0.124 (0.581), Chi² = 1.43 (0.96); P6₂22; for all data from 99–1.9 Å, Rmerge = 0.123 (0.69), Chi² = 1.55 (1.35), values for last resolution shell given in parentheses); therefore, initial phasing trials were performed in space group P6₂22 and P6₃22. It was possible to locate 9 anomalous scatterers, 8 seleniums, and 1 zinc, corresponding to 1 hSDH monomer using the automated heavy atom location algorithm (Grosse-Kunstleve and Brünger, 1999) implemented in the Crystallography and NMR System (CNS) (Brünger et al., 1998). Maximum likelihood single-wavelength anomalous diffraction (SAD) phasing and density modification by solvent flipping (Abrahams and Leslie, 1996) with CNS generated an electron density map that showed excellent density for an hSDH monomer in space group P6₃22. The functionally relevant hSDH tetramer could be generated by application of the two crystallographic 2-folds.

Packing considerations, the presence of additional electron density, and strong positive peaks in the log-likelihood gradient maps suggested that additional hSDH molecules were present in the asymmetric unit. However, it was not possible to place another molecule without serious symmetry overlaps. Therefore, the translational NCS operator was used to generate a second set of anomalous scatterer sites and SAD phasing repeated in the lower symmetry P6₃ cell. Strong positive peaks in the log-likelihood gradient map unambiguously identified additional sites corresponding to two additional hSDH monomers. The final SAD phasing used diffraction data to 1.9 Å indexed in space group P6₃, 36 selenium sites, and 4 zinc sites (Table 1). After density modification with a solvent content of 65%, excellent electron density was seen for all 4 molecules in the asymmetric unit, which consisted of 2 hSDH dimers related by the translational NCS. Application of the crystallographic symmetry results in two tetramers.

The experimental map was of such high quality that much of the amino acid sequence could be deduced directly from the electron density. Analysis of the selenium site locations and the electron density map indicated that residue 185 was in fact a selenomethionine, rather than a glutamic acid. We used the Cu trace of a theoretical model of sheep liver sorbitol dehydrogenase (PDB entry 1SDG, based on horse liver alcohol dehydrogenase; Eklund et al., 1985) as a starting point for model building of one monomer. NAD⁺ CP-166,572, the catalytic zinc, and residues 4–356 of each monomer were unambiguously positioned in the map using the program O (Jones et al., 1991).

Refinement

The structure was refined using the MLHL maximum likelihood in CNS (Adams et al., 1999). Crossvalidation used 5% of the reflections selected prior to the start of refinement. One molecule was manually reﬁned in O (Jones et al., 1991), and the other three were regeneratated by application of noncrystallographic symmetry. Standard CNS protocols of rigid body re ﬁnement, overall B factor re ﬁnement of each monomer, grouped B factor re ﬁnement of main chain and side chain atoms, minimization, torsional dynamics, individual B factor re ﬁnement, and automatic water picking were used. Prior to torsional dynamics, values for A' and F' were refined for the selenium and zinc atoms. These anomalous scattering contributions were included in structure factor calculations in all subsequent steps. The structure was refined using all data at the peak wavelength between 99.0 and 1.9 Å and resulted in a final free R value of 0.183 and R value of 0.211 (Table 1). The starting models for the native enzyme and SDH/NAD⁺ complex consisted of the coordinates for SDH/NADH/CP-166,572 complex with ligands removed. Refinement of the native enzyme and SDH/NAD⁺ complex used the standard methods described above and resulted in final free R values of 0.233 and 0.222 and R values of 0.200 and 0.195, respectively (Table 1).

Acknowledgments

We thank Professor Jan-Olov Höög for helpful discussions in the early stages of this project and Andrew P. Seddon for his continued support.

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Accession Numbers

Atomic coordinates have been deposited in the Protein Data Bank under ID codes 1PL7 for the apo enzyme, 1PL8 for the complex with \( \text{NAD}^+ \), and 1PL6 for the hSDH/NADH/CP-166,572 complex.